The nature and nurture of female receptivity

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Different genes influence virgin and mated female receptivity in Drosophila melanogaster: a genome wide association study

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Abstract

Female receptivity, the likelihood to accept mating, varies between individuals in part due to genetic variation. Sexual receptivity is also affected by the mating state of the female as virgin females are generally more receptive than mated females. Because the costs and benefits of mating differ between virgin and mated females, we hypothesised that different genes influence virgin and post-mating sexual receptivity. We tested this hypothesis in *Drosophila melanogaste*r by mapping genetic variation associated with three female receptivity phenotypes in a Genome Wide Association Study (GWAS) of the core lines of the *Drosophila* Genetic Reference Panel (DGRP). The virgin latency of these different lines is uncorrelated to two highly correlated post-mating phenotypes (latency to first remating and number of copulations within 24h). Similarly, virgin and post-mating receptivity phenotypes have only one gene in common, while the two post-mating phenotypes have over 50% overlap in genetic variation. The product of genes associated with virgin and post-mating receptivity are expressed in three main systems: digestive, reproductive and nervous systems. For post-mating receptivity, central nervous system genes are implicated in learning and memory and the mushroom bodies, suggesting that integration of different cues is an important feature of post-mating receptivity. Functional study of these genes using RNAi targeted to the Mushroom bodies revealed that *Pde8*, a phosphodiesterase, influences post-mating but not virgin sexual receptivity. We conclude that variation in female virgin and post-mating receptivity rely on different genes and mechanisms.

Keywords

Female sexual receptivity, virgin receptivity, post-mating receptivity, DGRP, GWAS, *Drosophila melanogaster*, mushroom bodies.

Introduction

Sexual receptivity generally differs in virgin and mated females. Mating brings clear fitness benefits to virgin females as it is necessary to ensure fertilization (Kokko and Mappes, 2005). Not mating is even suggested to be costly beyond this obvious fertilization function as virgin females have a decreased lifespan compared to mated females (Markow, 2011). Mating is, however, costly to females, because of the energy investment in egg production, as well as an increased risk of lifespan reduction, physical damage and immunological challenges caused by males (Bateman, 1948; Chapman et al., 1995; Kamimura, 2007; Kuijper et al., 2006; Rice et al., 2006; Schwenke and Lazzaro, 2017). The balance in costs and benefits of high and low virgin receptivity differs depending on context. In a low social density context, for example, low receptivity can be costly as it makes a female more likely to reject the only mate she encounters and thereby increase her chances of dying without offspring (Kokko and Mappes, 2005), whereas this same level of receptivity does not cause any problems in a high social density context where the chances of finding a mate are very high. Different context can, therefore, promote different levels of virgin receptivity, leading to genetic variation in the level of virgin receptivity. Already mated females can benefit from additional copulations, for instance to ascertain fertilization, offspring genetic diversity and to trade-up sires (Arnqvist and Nilsson, 2000; Gowaty et al., 2010; Jennions and Petrie, 2000; Priest et al., 2008). However, additional mating is not directly necessary as one mating with a fertile male is sufficient to fertilize all available eggs (Sirot et al., 2011) and can be disadvantageous due to the costs of mating. The balance of these costs and benefits for multiple mating (or post-mating receptivity) is affected by context. High post-mating receptivity, for example, increases offspring production in a context with high availability of food, because food promotes the production of eggs and increase the chances for offspring survival (Becher et al., 2012; Bownes et al., 1988; Lee et al., 2008; Terashima, 2004), whereas females can produce offspring with only one mating in any given context. Both high and low post-mating receptivity can thus co-exist, leading to genetic variation in the level of post-mating receptivity. However, how female receptivity, both virgin and mated, is determined is unclear. The model organism *Drosophila melanogaster* shows a clear distinction in female virgin and post-mating receptivity offering a model system in which to study phenotypic variation to further our understanding of the genetic mechanisms that influence female receptivity.

Female sexual receptivity in *Drosophila melanogaster* is influenced by both external and internal factors. External factors are, for example, food or yeast availability (Fricke et al., 2010; Gorter et al., 2016; Harshman et al., 1988) and the presence and diversity of conspecifics (Billeter et al., 2012; Gorter et al., 2016; Krupp et al., 2008; Laturney and Billeter, 2016). Most internal factors, involved in virgin receptivity, are part of neuronal networks involved in responding to the male's courtship advances (Aranha et al., 2017; Bussell et al., 2014; Kurtovic et al., 2007; Lebreton et al., 2017b; Sakurai et al., 2013; Zhou et al., 2014), or link receptivity to nutritional state (Lebreton et al., 2017b; Sakai et al., 2009; 2014; Watanabe and Sakai, 2015). Post-mating receptivity has also been connected to the

genetic background of the female (Billeter et al., 2012; Krupp et al., 2008; Kuijper and Morrow, 2009; Newport and Gromko, 1984) and selection studies have shown a genetic basis for the time between first and second mating, remating latency, that can be selected for (Fukui and Gromko, 1991a; Giardina et al., 2011; Gromko and Newport, 1988a; 1988b; Lawniczak and Begun, 2004; Linder and Rice, 2005; Wigby and Chapman, 2004). Specific genes identified for post-mating receptivity are expressed in the female reproductive tract (Giardina et al., 2011), and associated with immune response (Lawniczak and Begun, 2004) or involved in sensing environmental conditions (Gorter et al., 2016; Wigby et al., 2011). These findings suggest a more influential role of environment on post-mating receptivity than virgin receptivity. This leads to the hypothesis that the genetic variation involved in virgin and post-mating receptivity might differ.

To add to the understanding of the mechanisms underlying female sexual receptivity, both virgin and mated, we investigated natural variation using a genome wide association (GWA) approach to identify new candidate genes for virgin and mated female receptivity that can be functionally tested. The *Drosophila* Genetic Reference Panel (DGRP) is a panel of 205 isofemale lines collected from one single population, whose genomes have been fully sequenced (Huang et al., 2014; Mackay et al., 2012). By phenotyping each of these lines, phenotypic variation in female sexual receptivity can be mapped to genetic variation. Several phenotypes vary quantitatively between lines, enabling the genome wide mapping of the genetic variation underlying these quantitative phenotypic differences. This has been achieved for phenotypes such as male courtship (Gaertner et al., 2015), aggression (Rohde et al., 2017; Shorter et al., 2015), food intake (Garlapow et al., 2015) and virgin female longevity (Ivanov et al., 2015). With a core set of 40 DGRP lines capturing most genetic variation in this library, it is possible to identify alleles associated with an observed phenotype (Chow et al., 2013; Toshima et al., 2014; Zwarts et al., 2015). Using all 40 lines of the DGRP core set, mushroom body morphology could be linked to 139 candidate genes of which only 8 were previously identified (Zwarts et al., 2015). With 39 lines, female influence on male-male sperm competition was associated with a high percentage of neurological genes (Chow et al., 2013). Finally, with only 36 lines of the DGRP core set, variation in response to amino acid food preference was mapped in high responding and low responding lines (Toshima et al., 2014). These examples show that the relatively small DGRP core set is powerful enough to map candidate genes involved in the phenotype of interest. Here, we use this approach to map genes involved in female sexual receptivity, by determining the genetic variation for virgin and post-mating receptivity.

We found variation for both virgin and mated female sexual receptivity by phenotyping 34 DGRP core set lines. Two measurements for post-mating receptivity, mating frequency and remating latency, are correlated in these lines, while virgin mating latency is not. We found considerable overlap in genes between the two post-mating measurements and only one gene occurred both in virgin and post-mating receptivity. The genes identified can be subdivided into three systems based on expression pattern and known function; digestive, reproductive and central nervous systems. To test the hypothesis that specific genes identified

in our GWAS are important in determining post-mating receptivity, we performed an RNA interference study on candidate genes isolated in the GWAS. With this approach, we showed that *Pde8* expression within the mushroom bodies is necessary for post-mating receptivity. This study, therefore, shows that virgin and mated female receptivity have distinct genetic backgrounds and shows that genes for sexual receptivity can be identified by GWAS, which can then be functionally tested through gene manipulation.

Material and methods

Drosophila stocks

Thirty-four isofemale lines (annotated with identity number RAL-XXX) of the core set of the fully sequenced *Drosophila* Genetics Reference Panel (DGRP) were used (Ayroles 2009, Mackay 2012) for the genome wide association study obtained from Bloomington Stock Center. For the RNAi validation of specific genes the following UAS-RNAi lines were obtained from Vienna *Drosophila* Resource Center (VDRC stock, (Dietzl et al., 2007)): y^-,w^-;UAS-CG10205-RNAi;+ (#107665), y^-,w^-;UAS-*vn*-RNAi;+ (#109437), y^-,w^-;UAS-*tau*-RNAi;+ (#101386), w^{1118};UAS-*Dhc64c*-RNAi;+ (#28054), y^-,w^-;UAS-*CrebB-17a*-RNAi;+ (#101512), y^-,w^-;UAS-*lilli*-RNAi;+ (#106142), y^-,w^-;UAS-*Pde8*-RNAi;+ (#101413), y^-,w^-;UAS-CG11170-RNAi;+ (#106277), w^{1118};UAS-CG14741-RNAi;+ (#102648), y^-,w^-;UAS-*Eip75b*-RNAi;+ (#108399) and y^-,w^-;UAS-attP;+ (#60100). Y^-,v^-;UAS-*shot*-RNAi;+ (#28336) was obtained from Bloomington Stock Center. The UAS-RNAi lines or the insertion site control (y^-,w^-;UAS-attP;+, (Dietzl et al., 2007)) were expressed with either y^-,w^-;*nSyb*-gal4;+ (#51635, (Venken et al., 2011)) or w^{1118};OK107-gal4;+ (#854, (Aso et al., 2009)) obtained from Bloomington Stock Center. Wild-type y^-,w^- and w^{1118} were used as background controls in control crosses. All test males were wild-type *Canton-S* (*CS*) to restrict behaviour and genetic variation to females.

Rearing conditions

Flies were reared in a 12:12 hr light/dark cycle (LD 12:12), lights on at 09:00 local time (*Zeitgeber time* 0), in 25°C. Rearing was done on food medium containing agar (10g/L), glucose (167mM), sucrose (44mM), yeast (35g/L), cornmeal (15g/L), wheat germ (10g/L), soya flour (10 g/L), molasses (30 g/L), propionic acid (5 ml of 1M) and Tegosept (2g in 10ml ethanol). This medium is called "Fly food" in this report. Virgin adults were collected using CO_2 anaesthesia and aged in same-sex groups of 20 in food vials for 5-8 days. Flies were raised in the same light and temperature conditions as rearing.

Phenotype characterization

To obtain a measure of female post-mating receptivity, we performed mating assays as described in (Gorter and Billeter, 2017). All assays were done in small Petri dishes of 35x10 mm with 3 ml of fly food poured on the bottom and the same light and temperature conditions as during rearing. Red light was utilized to monitor behaviour during the dark

phase. The test subject(s), virgin female of indicated genotype, and corresponding male, wild-type *Canton-S*, were aspirated into the Petri dish. All experiments began in the active phase in the afternoon between *Zeitgeber time* 7 and 9. Webcam cameras (Logitech B910 webcam using the SecurityMonitor Pro software [Deskshare]) took pictures of the dishes at 2-min intervals for 42 hour to score virgin mating latency, number of matings in 24 hours and latency to 1st remating (Billeter et al., 2012; Krupp et al., 2008). Virgin mating latency was scored as the start time of first mating minus the start time of the experiment (moment both male and female are in the arena and the arena is placed under the camera). The remating latency was defined as start time of second mating minus start time of first mating. If no second mating occurred in the first 24 h, the data were checked up to 42 h. If still no second mating occurred, remating latency was defined as 2500 min. Flies that didn't mate at all were excluded from all abovementioned analyses. Any outliers were excluded based on [based on average±(standard deviations*3)]. This was less than 10% of the replicates.

GWAS correlations

Measures of virgin latency, number of copulations in 24h and remating latency, were uploaded independently in the DGRP2 analysis pipeline at http://dgrp.gnets.ncsu.edu/ (Huang et al., 2014; Mackay et al., 2012), which generates an output of a Genome Wide Association Study with all the genetic variation sites and their association to the uploaded phenotype stated as p-value. From the output files, we continued with the top associated Single Nucleotide Polymorphism (SNP) list which contains the variation sites with the most significant association. This list might contain false positives due to the high number of statistical associations, but it is the cut-off line used in most DGRP studies (Mackay et al., 2012) and provides a useful starting point for functional analyses of associated genes (Akhund-Zade et al., 2017).

Statistical analysis

Variation between genotypes was statistically determined with mixed effect models with the lme4 package in R (Team, 2015). Genotype was included as the main fixed effect. Whether genotype was an explaining variable was determined based on log-likelihood ratio tests between the model including and excluding genotype as fixed effect, these results are recorded in supplementary table 5 and as p-value in the graphs of DGRP phenotypes. Normal distribution of residuals was inspected visually and data was tested on homogeneity of variances with Levene's test. Whenever the data did not pass either one of the assumption, a log transformation was performed, if necessary followed by a Z transformation. The correlation between phenotypes in the DGRP dataset was visualised with the ggpubr package and analysed with Pearson correlation, the results are summarised in supplementary table 5 and reported in the figures. The data were first tested for normality with a Shapiro-Wilk test and log-transformed to comply to normality whenever necessary. For the functional testing of genes with RNA interference all genotypes were additionally post-hoc tested with a mixed effects model including two genotypes at the time when initially genotype was determined as

explaining variable, details are recorded in supplementary table 5 and significance is displayed in the figures with letters. When genotype is not a determining factor in the model, its p-value as main effect is reported in the figures.

Results

Phenotypic variation in virgin and post-mating receptivity are two distinct phenomena

We tested DGRP virgin females from 34 lines for 24 to 42 hours housed with a *CS* tester male and scored both their virgin and post-mating sexual receptivity. First, we measured virgin receptivity as the time to achieve mating from the start of the assay. Virgin latency is highly variable between strains with averages between 5 min and 4 hours (figure 1A). Second, we measured the post-mating receptivity in these lines as two phenotypes: number of copulations in 24h and latency to first remating. The mating frequency is variable between 1.5 and 2.8 mating in 24h (figure 1B). Time to first remating is correspondingly variable with the least receptive lines taking up to 25h to remate, while the most receptive lines remated on average within 10h (figure 1C).

Virgin and post-mating receptivity do not correlate; lines with high virginal receptivity are not more likely to have high or low post-mating receptivity (figure 1D and 1E). The two measurements of post-mating receptivity, number of copulations and latency to first remating, are negatively correlated (figure 1F). This means that the line with the lowest number of copulations also takes the longest time between first and second mating and vice versa. Variation in post-mating receptivity can thus not be predicted from variation in virgin receptivity, while the number of copulations can be predicted from the latency to first remating.

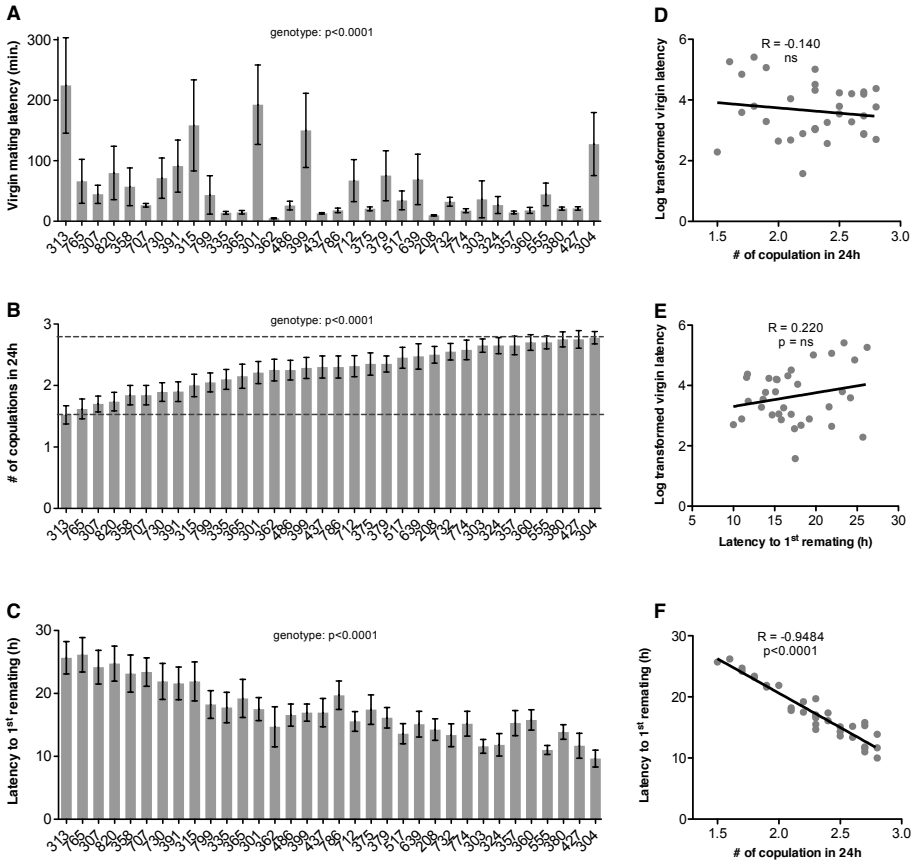

Figure 1: Virgin and post-mating receptivity are independently variable phenotypes in the DGRP core set Bar graphs with errors bars SEM of virgin mating latency in minutes (A), number of copulations in 24h with dashed line depicting range of variation of averages (B) and latency to first remating in hours up to 42h (C) of 34 DGRP core set lines with RAL numbering on x-axis in order of low to high mating frequency, tested with CS males. P-value of genotype effect reported from mixed effects models. N=18-22. Correlation plots for average values per line of log transformed virgin latency and number of copulations (D), log transformed virgin latency and latency to first remating (E) and latency to first remating and number of copulations (F). Correlation coefficient and p-value of Pearson correlations are indicated above the graphs.

Top associated genes of virgin and post-mating receptivity differ

To characterize the genetic variation underlying line variation in both virgin and post-mating receptivity, we used the DGRP2 internet tool (Huang et al., 2014; Mackay et al., 2012) to perform genome wide association (GWA) analyses for the three measurements previously described; virgin mating latency, number of copulations in 24h and latency to first remating (supplementary table 1). With this approach, the top hits provided by the GWA analyses included variation in 54 annotated genes for virgin receptivity and 38 for post-mating receptivity of which some genes had several variation sites associated to the phenotypes (supplementary table 2 and 3). Additionally, variation sites were found in intergenic regions for both types of receptivity (data not shown). The variation sites in annotated genes implicate a function for the protein product of these genes in female receptivity, while variation in intergenic regions most likely indicates regulatory sites affecting the spatial, temporal or quantitative expression of genes important to female sexual receptivity. Most variation sites identified in annotated genes were single nucleotide polymorphisms (SNPs), but also included six deletions ranging from 1 to 24 base pairs and one insertion of 8 base pairs, as compared to a reference genome. The SNP variation sites include all kinds of positions in the genome from intron, upstream, downstream of the genes to synonymous and non-synonymous coding sites. This suggests a role for regulation or changes in levels of expression, rather than actual changes in the gene products as only a minority leads to changes in the amino acid sequence of the coding region.

The phenotype for virgin receptivity provided the most variation sites with p-value thresholds of 10^{-5}. This is due to the high magnitude of variation between the different lines. However, this phenotype also shows the highest variation within lines, making it less predictable. Of the phenotypes for post-mating receptivity, latency to first remating had the highest number of significant ($p<0.05$) SNP sites associated compared to number of copulations. While the level of significance was higher for mating frequency (supplementary table 3). These differences can be explained by the range of variation between lines, for remating latency the range is larger (ranging from 10h to 25h) which creates the opportunity to detect more different associations, while the limited variation in number of copulations (ranging from one to three copulations) makes it possible to group lines with the same phenotype leading to stronger associations when the genotypes coincide.

Strikingly, of the 54 annotated genes for virgin receptivity, only one gene was shared with the two measurements of post-mating receptivity, namely the gene *Kirre* (figure 2). Of the 37 genes for post-mating receptivity, only one was specifically associated with number of copulations and 16 were specifically associated with latency to first remating (figure 2). The remaining 20 associated genes were common to both phenotypes (figure 2). These findings are in keeping with the difference in correlation between phenotypes and show that virgin receptivity depends on variation in different genes than post-mating receptivity.

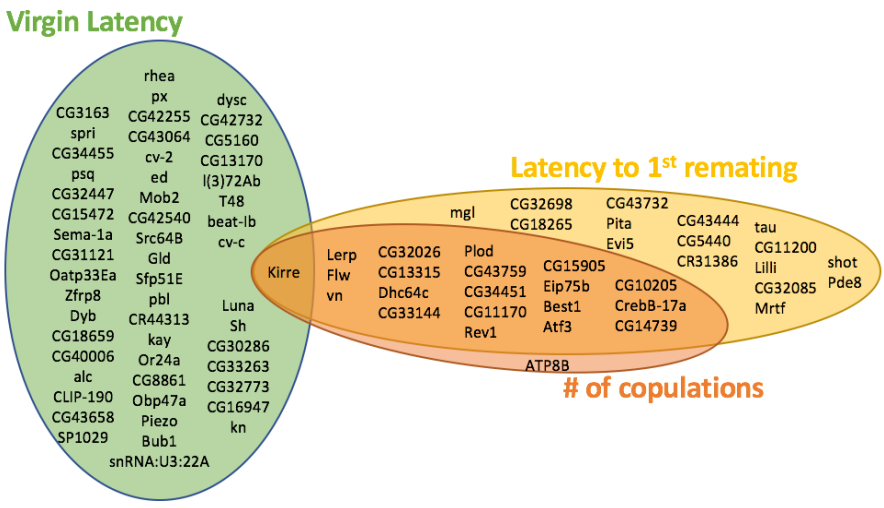

Figure 2: Genetic variation associated with mated and virgin receptivity differs Representation of annotated gene lists from GWA analyses of the three female receptivity phenotypes; virgin mating latency in minutes, latency to first remating in hours and number of copulations in 24h. All hits in annotated genes are included and the overlap between the three phenotypes is visualized.

Top associated genes of female receptivity fit into three main themes

To further explore the functional significance of the candidate genes revealed by the GWAS, we summarised their adult expression pattern based on data from the gene expression database flyatlas (www.flyatlas.org, supplementary table 4, (Chintapalli et al., 2007)) and searched for gene function (www.flybase.org). This survey revealed three main systems (figure 3A and 3B). The first is expression in the reproductive system or sex specific functions. The second is the presence of several genes associated with food processing or with expression within the digestive system. The third are genes associated with the central nervous system and its development. Interestingly, even though virgin and post-mating receptivity only share one gene, all three systems are represented in both phenotypes (figure 3A and 3B). The appearance of the three systems is equal between the two phenotypes, but the distribution between genes involved in only one, two or all three categories differs with genes for post-mating receptivity being more often involved in several categories. The three systems seem, therefore, more tightly linked in post-mating receptivity (figure 3B) than virgin receptivity (figure 3A).

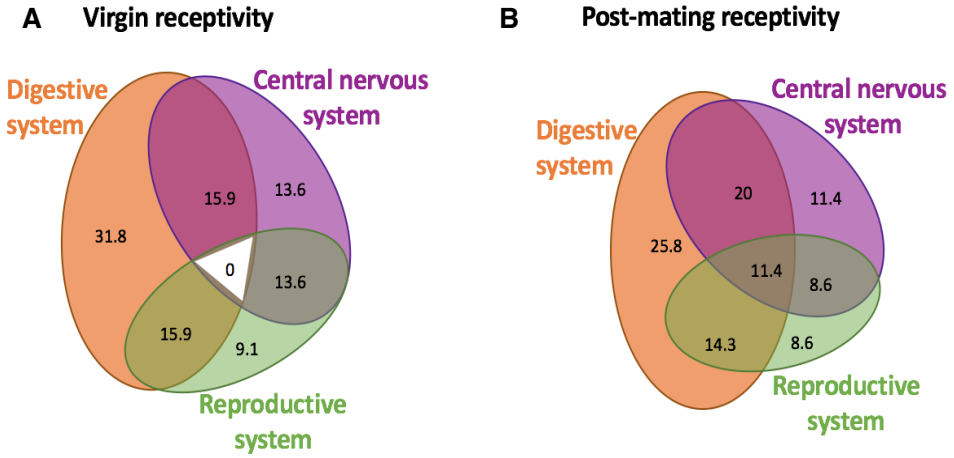

Figure 3: Genes associated with female receptivity can be divided in three main themes Representation of gene characterization based on tissue expression. Most tissues the genes were expressed in could be divided into digestive system, reproductive system and the central nervous system. This division represents 48 of the 54 genes for virgin receptivity and 36 of the 37 genes for post-mating receptivity. Number of genes belonging to each category (food, central nervous system, sex specific or any combination of these) are noted as percentages of the total number of genes found for that phenotype.

The third system, representing genes involved in the central nervous system, is somewhat of a surprise. Sexual behaviours are often thought to be innate behaviours that involve central processing, but they are usually not expected to involve memory and integration. We hypothesize that this is one of the differences between virgin and mated receptivity. Literature review of the genes associated by our GWAS with virgin receptivity that are expressed in the central nervous system shows that 61% of these genes are associated with olfaction (Arya et al., 2015) and 22% are associated with memory and sensory integration (supplementary table 2). This suggests that the central nervous system genes in virgin receptivity are involved in direct responses and not memory and integration processing. Contrary to virgin receptivity, only 37.5% of central nervous system genes associated with post-mating receptivity phenotypes are linked to olfaction, while 31.3% are associated with memory and sensory integration (supplementary table 3). Genes like *crebB-17a* and *tau* have a well-documented role in associative memory (Ihara et al., 2016; Santa-Maria et al., 2015; Yin et al., 1995). Additionally, the genes *shot* and *dhc6c* are involved in mushroom body development (Liu et al., 2000; Reuter et al., 2003), a central brain region necessary for learning and memory (Davis, 1993; Owald and Waddell, 2015). This suggests a prominent role for processing previous and new information in post-mating receptivity and fits with our starting hypothesis that post-mating receptivity considers more environmental information than virgin receptivity. We thus decided to further validate the involvement of these learning and memory genes in post-mating receptivity to solidify the hypothesis that memory and integration genes are involved in mated female receptivity.

Functional test of candidate genes expressed in the central nervous system associated with post-mating receptivity

We functionally tested genes with expression in the central nervous system and in particular with function in memory and sensory integration processes. We shortlisted 11 candidate genes from supplementary table 3. Nine of these genes are expressed in the adult brain, thoracicoabdominal ganglion or head (supplementary table 3) and two caught our interest based on their predicted functions in learning and memory.

The first nine genes include *crebB-17a*, *tau*, *shot*, *Dhc6c* and *Pde8* which have, next to other functions in cell development, been implicated in learning, memory or brain development (Ihara et al., 2016; Liu et al., 2000; Reuter et al., 2003; Santa-Maria et al., 2015; Yin et al., 1995). *ATP8B* (CG14741) was included for its role in the sensory perception of smell (Liu et al., 2014). *Eip75b* was included first because of its abundance in the brain and thoracicoabdominal ganglion and second for its link to female reproduction through ecdysone (Bialecki et al., 2002; Buszczak et al., 1999; Johnston et al., 2011). CG11170 and CG10205, expressed in the brain, but without known function were included because of their appearance in both lists and CG11170 included the only non-synonymous coding variant for post-mating receptivity. From the two not directly obvious choices, the gene *vein* (*vn*) came up expressed in the eye, heart and tubule, but is also involved in brain and nervous system development (Page, 2003; Sepp and Auld, 2003) and olfactory learning in larvae (Rahn et al., 2013). The gene *lilli*, expressed in the testes, heart and eyes, has been previously implicated in learning and memory (Winbush et al., 2012) and olfactory behaviour (Arya et al., 2015; Sambandan et al., 2006).

To functionally test these 11 candidates, we knocked down their expression using RNA interference targeted to the nervous system of females with *nSyb*-gal4, driver of neuron specific vesicle machinery *synaptobrevin* (*nSyb*) ((Venken et al., 2011), figure 4A), and quantified post-mating receptivity. None of the knock-downs of these genes had a clear effect on mating frequency as compared to their control (figure 4B-L), nor do they show an effect on latency to first remating (figure 5A-K). In some cases the RNAi line showed a different behaviour compared to one of the controls (figure 4B,C,G,H,J and 5B,C,E-J), in some cases there was an effect of genotype, but post-hoc analysis did not confirm (figure 4D,F,I) and in some cases there was no effect of genotype (figure 4K,L and 5J,K). From these results, we can only conclude that any effect these genes might have on female post-mating receptivity cannot be attributed to their expression in the whole central nervous system, or that these genes were not silenced effectively by our RNAi approach.

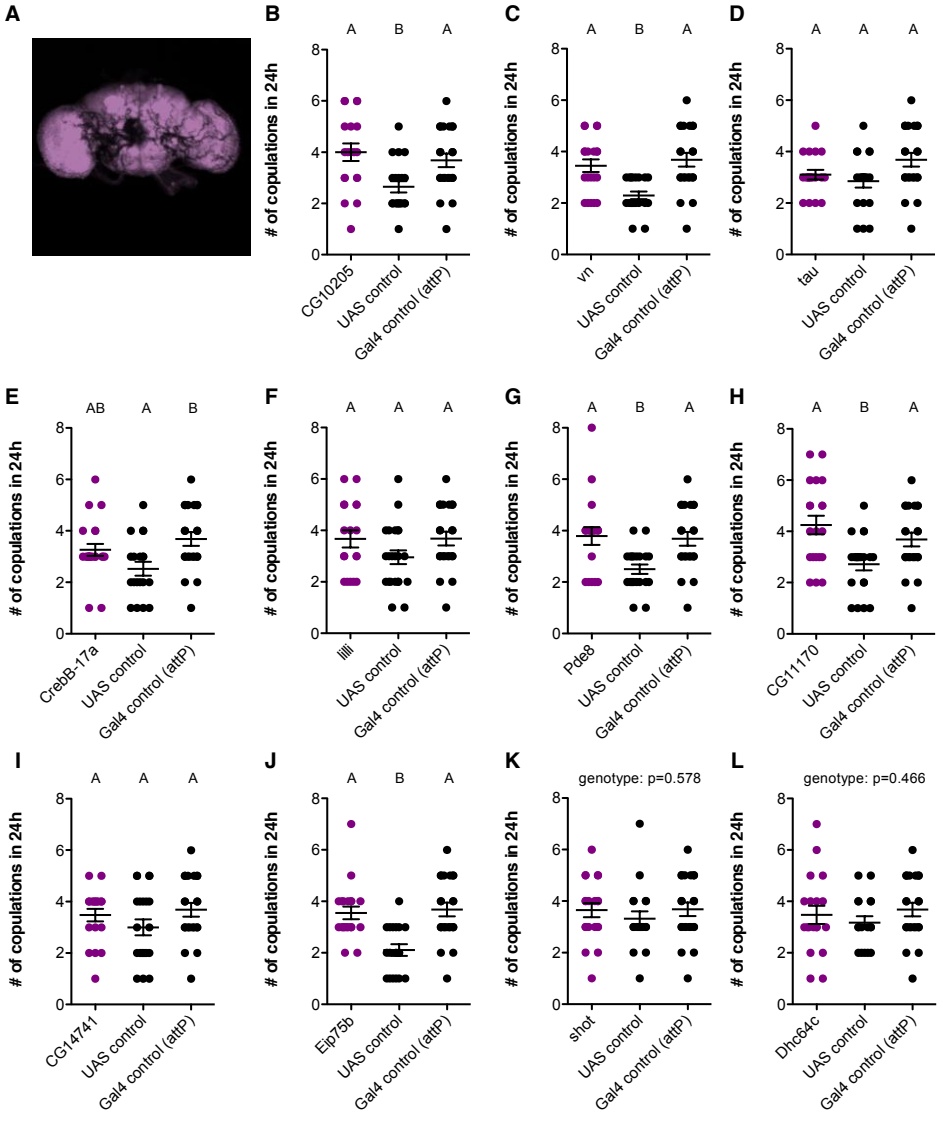

Figure 4: Knockdown of none of the candidate genes in the central nervous system affects mating frequency Expression of UAS-GFP driven by nSyb-Gal4 visualized with Leica fluorescence microscope with highest magnification of 0.63x lens (A). Dot plots with average and error bars SEM of number of copulations of females with nSyb-Gal4 driving UAS-RNAi and the two controls for CG10205 (B), vn (C), tau (D), CrebB-17a (E), lilli (F), Pde8 (G), CG11170 (H), CG14741 (I), Eip75b (J), shot (K) and Dhc64c (L), tested with CS males. P-value of mixed effects models reported when no genotype effect was present. Post-hoc analyses reported with different numbers showing significant differences. N=17-22.

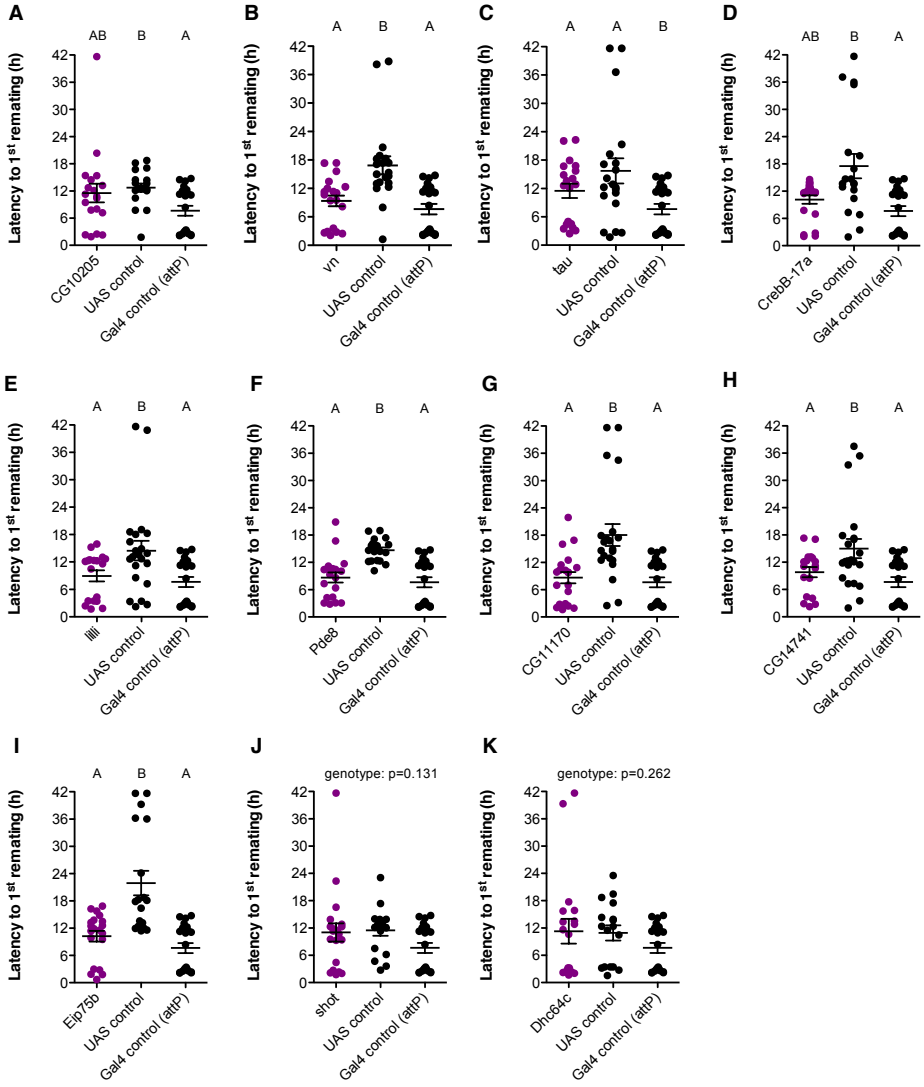

Figure 5: Knockdown of none of the candidate genes in the central nervous system affects remating latency Dot plots with average and error bars SEM of time to first remating of females with *nSyb*-Gal4 driving UAS-RNAi and the two controls for CG10205 (A), *vn* (B), *tau* (C), *CrebB-17a* (D), *lilli* (E), *Pde8* (F), CG11170 (G), CG14741 (H), *Eip75b* (I), *shot* (J) and *Dhc64c* (K), tested with *CS* males. P-value of mixed effects model reported when no genotype effect was present. Post-hoc analyses reported with different numbers showing significant differences. N=17-22.

***Pde8* expression in the mushroom bodies influences post-mating receptivity**

To continue the analysis of the central nervous system candidate genes for female post-mating receptivity, we aimed to assess whether the effect might be localised to a specific part of the brain which might have been overruled by the genes' lack of expression in other parts of the central nervous system in the previous functional interference analysis. To determine the pattern of expression for each of the 11 genes in the central nervous system, we searched the flylight database, which is a database of characterised Gal4 lines with different promotor regions of known genes (Jenett et al., 2012; Jory et al., 2012; Li et al., 2014; Manning et al., 2012; Pfeiffer et al., 2008). Only 6 of our candidate genes are represented in the Flylight database and they did not show specific brain areas in common (supplementary table 4). Based on literature review, we decided to focus on the mushroom bodies for its role as memory and integration site of olfactory information (Heisenberg, 2003). Additionally, it is one of the sites of expression of several of our candidate genes.

We knocked down the candidate genes with RNA interference in the mushroom bodies with *Ok107*-gal4, mushroom body driver with expression in all lobes ((Aso et al., 2009), figure 6A). The knockdown of one gene in the mushroom bodies of females showed a significant effect on both mating frequency and latency to first remating when tested with a *CS* male (figure 6G and 7F), namely *Pde8*. *Pde8* RNA interference induces decreased post-mating receptivity compared to the Gal4-control, but with an intermediate phenotype for the UAS-control (figure 6G and 7F). This result is, therefore, not conclusive, but it suggests a role for *Pde8* in promoting post-mating receptivity as the interference line shows decreased mating frequency and an increased latency to first remating.

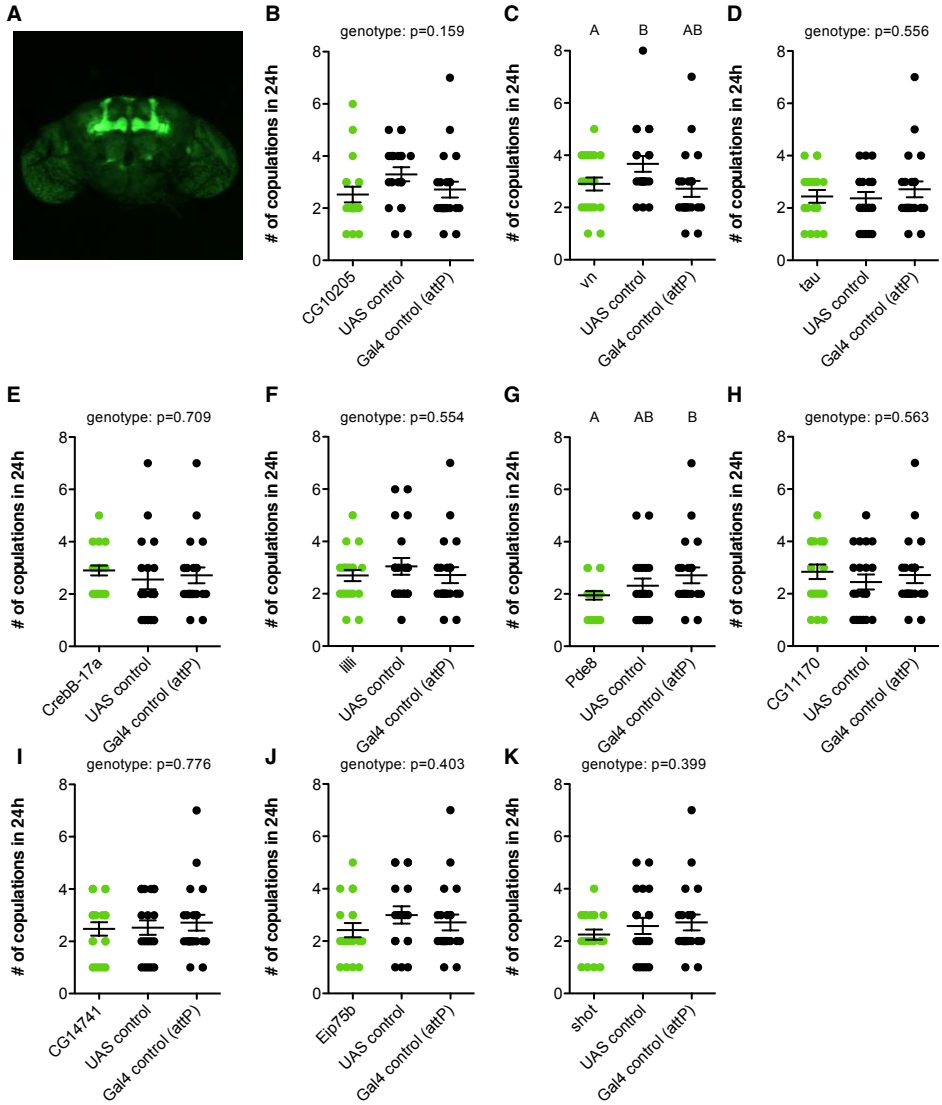

Figure 6: Knockdown of Pde8 in the mushroom bodies decreases mating frequency Expression of UAS-GFP driven by Ok107-Gal4 visualized with Leica fluorescence microscope with highest magnification of 0.63x lens (A). Dot plots with average and error bars SEM of number of copulations of females with Ok107-Gal4 driving UAS-RNAi and the two controls for CG10205 (B), vn (C), tau (D), CrebB-17a (E), lilli (F), Pde8 (G), CG11170 (H), CG14741 (I), Eip75b (J) and shot (K), tested with CS males. P-value of mixed effects model reported when no genotype effect was present. Post-hoc analyses reported with different numbers showing significant differences. N=18-21.

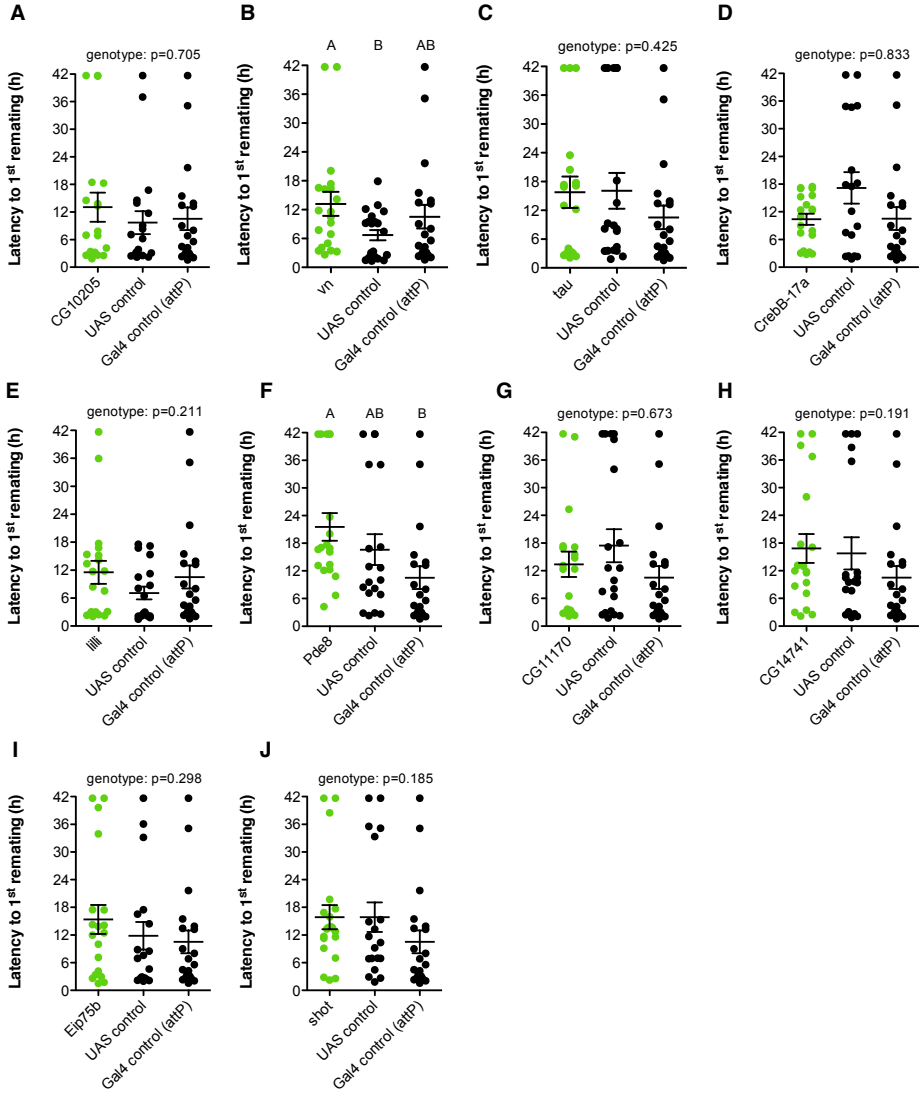

Figure 7: Knockdown of *Pde8* in the mushroom bodies increase remating latency Dot plots with average and error bars SEM of time to first remating of females with *Ok107*-Gal4 driving UAS-RNAi and the two controls for CG10205 (A), *vn* (B), *tau* (C), *CrebB-17a* (D), *lilli* (E), *Pde8* (F), CG11170 (G), CG14741 (H), *Eip75b* (I) and *shot* (J) tested with *CS* males. P-value of mixed effects model reported when no genotype effect was present. Post-hoc analyses reported with different numbers showing significant differences. N=18-21.

Discussion

In this study, we have tested a core set of 34 DGRP lines for female sexual receptivity. We find variation among the different lines for three phenotypes: virgin mating latency, number of copulations in 24h and latency to first remating. Phenotypic variation in the post-mating phenotypes number of copulations and latency to first remating are negatively correlated, while latency to first mating is uncorrelated with these measures of post-mating receptivity. This indicates that virgin female receptivity has a different genetic basis than that of mated female receptivity, in line with our previous finding that the odorant receptor *Or47b* is necessary for mated but not virgin female receptivity (chapter 5).

To generate candidate genes and further understand genetic variation underlying female receptivity, we obtained GWA analysis on the virgin and post-mating female sexual receptivity phenotypes. Of note is the fact that we only used 34 of the 200 lines to obtain these results. Although we found considerable variation within the DGRP core set, there is a higher chance of false positives than when using the full set. Additionally, there is a high risk of missing relevant variation, due to this small genetic sample size, giving more weight to individual cases and loss of strength for small effect sizes. All three phenotypes provided a list of significantly associated SNP variation sites with a total of 54 annotated genes for virgin receptivity and 37 annotated genes associated with either one or both measurements of post-mating receptivity. From these genes, only 1 came up for all three measurements and 20 of the post-mating receptivity genes had significant SNP variation sites for both measurements. These observations support the hypothesis that virgin receptivity is an independent phenomenon and that the two measures of post-mating receptivity quantify two correlated phenotypes.

Most SNP sites identified are in introns, synonymous and non-synonymous coding and 3' and 5' UTR regions. The rate of intron incidence is not surprising given there are more SNP variation sites characterized as introns in the DGRP lines than any of the other location types (Mackay and Huang, 2017). This suggests that the variation in the genes identified is most likely not due to changes in the protein, but rather its expression through SNP variation in regulatory regions (Mackay and Huang, 2017). Another explanation for these sites is that these SNPs hitchhiked on genes of interest, meaning that not the SNP per se, but rather its approximate location might be of importance (Franssen et al., 2015). For these reasons, we considered this list of SNP variation sites as hypotheses generators on the involvement of associated genes, rather than direct proof of genes determining receptivity.

Many of the genes identified are associated with olfaction. For example, *ATP8B* is required for odorant receptor function (Liu et al., 2014), *vn* is necessary for olfactory learning (Rahn et al., 2013) and *lilli* is associated to odour-guided behaviour (Arya et al., 2015; Sambandan et al., 2006). Additionally, a high incidence of genes came up in a previous GWAS on olfaction (Arya et al., 2015). This is not necessarily surprising as olfaction is an important sensory modality for female flies during courtship ((Gorter et al., 2016; Markow, 1987), chapter 4). What is interesting about this result is that the DGRP library often identifies variation related to chemosensation, like variation in cuticular hydrocarbon profiles

(Dembeck et al., 2015) and olfactory behaviour towards different odours (Arya et al., 2015; Brown et al., 2013; Dembeck et al., 2015; Swarup et al., 2012). Naturally this can be explained by the great importance of odours in the ecology of fruit flies, but they can also make one wonder whether olfaction is a particularly important sensory modality specific for this population or that it might even be an artefact of inbreeding. The DGRP lines suffered considerable losses during inbreeding, starting out from 1500 isofemales and ending up with 205 viable lines (Mackay and Huang, 2017). Therefore, before putting any general conclusions onto these olfaction related hits, more functional testing and analysis of other genetic libraries are necessary to determine whether these are of general importance or specific to the DGRP.

The genes identified by our GWAS fit into three main systems; digestive, reproductive and the central nervous system. In this chapter, we were interested to explore the central nervous system and its integration sites. Females respond to both auditory and olfactory cues (Markow, 1987; Rybak et al., 2002) and might, therefore, need to integrate these and other different stimuli for the appropriate behavioural outcome. Especially for post-mating receptivity, as this is a more plastic behaviour than virgin receptivity, ranging from within a couple of hours (Billeter et al., 2012; Gorter et al., 2016; Krupp et al., 2008; Lefevre and Jonsson, 1962; van Vianen and Bijlsma, 1993) to first remating occurring only after one or several days (Lefevre and Jonsson, 1962) depending on assay type, female genotype and sperm available in female storage. Whereas about 90% of virgin matings occur within 2h after start of the experiment (Letsinger and Gromko, 1985; van Vianen and Bijlsma, 1993). There are two higher order brain regions for the integration of information that both receive input from olfactory stimuli and are involved in mating behaviour, namely the mushroom bodies and the lateral horn (Grosjean et al., 2011; Jefferis et al., 2007; Vosshall and Stocker, 2007). The lateral horn has been implicated in innate responses, including female virgin receptivity, and the mushroom bodies in learning and memory processes, including male courtship (Heimbeck et al., 2001; Heisenberg, 2003; Jefferis et al., 2007; Neckameyer, 1998; Schultzhaus et al., 2017). The higher occurrence of mushroom body functioning for genes associated to post-mating receptivity suggests that post-mating receptivity indeed requires more integration processing than virginal receptivity. Although, the mushroom bodies are not necessary for females to switch from a virgin to a mated state induced by Sex peptide (Fleischmann et al., 2001), they can still be involved in the modulation of post-mating sexual receptivity.

To understand the role of a subset of the central nervous system genes and their involvement within the central nervous system or mushroom bodies we have conducted an RNA interference study. Knockdown of 11 candidate genes in the central nervous system did not lead to clear effects on post-mating receptivity. This lack of effect can be explained by at least three hypotheses. One, the interference for these lines was incomplete and this resulted in a lack of effect simply due to these genes still being expressed. Two, neither of these genes affect the phenotype on their own, but rather they all have small effects, influence each other or they need to work with another factor in the genetic background of the DGRP lines that is

not present in the *nSyb*-Gal4>UAS-RNAi lines. Three, the genes have epistatic effects in different brain regions that compensate each other towards the net outcome of no change. The fact that we find differences with interference in one specific brain area supports the third hypothesis, but without knowing the expression levels after interference the first hypothesis cannot be excluded.

When the expression of each of these 11 genes was specifically silenced in the mushroom bodies, the down regulation of one gene lead to a decrease in post-mating receptivity, namely *Pde8*, phosphodiesterase 8. This is intriguing, because another phosphodiesterase, *Dunce*, functions in both short-term memory and female sexual receptivity and fertility (Bellen and Kiger, 1987; Qiu and Davis, 1993). From a homology study, it appears that these *Pde*'s are cAMP phosphodiesterases (Day et al., 2005), essential molecular components of the cAMP signalling cascade (Ganguly and Lee, 2013). Mutations of cAMP genes, normally expressed in the mushroom bodies, lead to abolished olfactory learning (Conolly et al., 1996; Sokolowski, 2001), both short- and long-term memory, due to changes in cAMP expression (Sokolowski, 2001). Pde's like *dunce* and *Pde8* could, therefore, influence learning and receptivity through cAMP modulation within the mushroom bodies.

Of note in relation to our results on the central nervous system, memory and mushroom body hits is the fact that some considerable variation in mushroom body development has been found in the core set of DGRP lines (Zwarts et al., 2015). This variation might have made the analysis prone to finding genes involved in this brain area even though they are not normally involved in the phenotype. It might suggest that some of the variation in mushroom body development might not be part of the natural variation, but rather a defect due to adaptation to laboratory conditions. Both explanations could be a reason why we did not find many effects when we manipulated these genes in lines with a different genetic background. More detailed analysis of the mushroom body and female receptivity is warranted in the future. At the very least, these results might now focus future research towards this brain area as a relevant centre for controlling female receptivity. Another, more general, downside of GWAS is that the study is focussed on genetic variation and core genes might be missed, because their involvement in the phenotype is constrained in plasticity due to their central importance in the development of the phenotype. An example of this is the gene *Or47b* that does not come up in this study, but it is of key importance in determining post-mating receptivity, whether it is very high receptivity (*Canton-S* females) or rather low (w^{1118} females, chapter 5).

Overall we showed that virgin and post-mating receptivity are two distinct phenotypes that vary under different genetic influences. We identified candidate genes for variation in female sexual receptivity and divided them in three themes. Even though virgin and mated receptivity had similar spread across these themes, it seems that the actual genes expressed in the central nervous system differ distinctly in their function. Virgin receptivity includes more olfaction associated genes and post-mating receptivity involves memory and integration processes in the mushroom bodies. Functional analysis of a subset of genes

involved in learning and memory shows a promising impact of *Pde8* in the mushroom bodies on post-mating receptivity. However, further analysis on this and other candidates is needed to understand the genetic and cellular basis of virgin and post-mating receptivity.

Authors and Contributors

J.A.G. and J.-C.B. designed and interpreted the study and wrote the manuscript. J.A.G. and R.D.dB performed the experiments. J.A.G. performed the statistical analyses.

Acknowledgements

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Supplementary Information

Genotype (RAL)	Virgin latency (min)	# of copulation in 24h	Latency to 1st remating (h)
208	9.85	2.5	855
301	192.6842105	2.2	1050.5
303	36.3	2.7	695.7
304	127.6111111	2.8	601.1
307	44.6	1.7	1450
313	224.5238095	1.5	1539.1
315	158.5555556	2	1314.2
324	26.85	2.7	709.9
335	14.1	2.1	1065.3
357	14.6	2.7	916.9
358	57.10526316	1.8	1389.3
360	18.05	2.7	946.3
362	4.85	2.3	881.8
375	20.6	2.4	1045.3
379	75.35	2.4	966.8
380	21.05	2.8	832
391	91.15	1.9	1295.4
399	150.1428571	2.3	1017.4
427	21.3	2.8	700.7
437	13.05	2.3	1017.4
486	26.1	2.3	993.6
517	34.5	2.5	815.5
555	44.35	2.7	660.5
639	69.10526316	2.5	906.4
707	26.57894737	1.8	1402.1
712	67.15789474	2.3	932.6
730	71.26315789	1.9	1314.7
732	32.3	2.6	801.9
765	66.04761905	1.6	1569.2
774	17.57894737	2.6	910.9
786	18.05	2.3	1182.2
799	43.5	2.1	1092.7
820	79.78947368	1.7	1484.6
365	14.9	2.2	1152.4

Supplementary Table 1 Phenotypes submitted to DGRP2 web tool Average values per genotype as they were used in the DGRP2 tool for GWA analyses.

Gene	FlyBase ID	Chromosome position	Gene position	P-value # virgin latency*	Tissue expression in adult fly'	Sexspecific"	Food related"	Central nervous system"	Hit in olfaction GWA?	Involved in learning and memory?
Sh	FBgn0003380	X: 17927949	Intron	1.07E-07	Eye, brain		x	x	no	yes
CG30286	FBgn0050286	2R: 17424155	Synonymous coding	1.83E-07	Salivary gland, fat body, hindgut		x			
CG33263	FBgn0053263	3L: 13416506	UTR 3 Prime	2.64E-07	Salivary gland, midgut fat body		x			
CG32773	FBgn0052773	X: 4287715; X: 4287358; X: 4287366	Intron	1.00E-06; 4.61E-06 4.61E-06	Ovary, salivary gland, eye	x	x			
CG3163	FBgn0034961	2R: 19917448	Non-synonymous coding	6.29E-07	Brain, crop, salivary glands		x	x	no	-
spri	FBgn0085443	X: 10448592	Intron	8.67E-07	Tubule, ovary	x	x			
CG34455	FBgn0085484	3L: 9353222; 3L: 9353221	Upstream	1.17E-06; 2.58E-05	unknown					
psq	FBgn0263102	2R: 6481719	Intron	1.35E-06	Ovary, whole fly, brain	x		x	yes	no
CG32447	FBgn0052447	3L: 21695807;	Intron and upstream	1.35E-06	Eye, hindgut, salivary gland		x			
CG15472	FBgn0029724	X: 4662378; X: 4662396	Downstream	1.35E-06	Testis	x				
Sema-1a	FBgn0011259	2L: 8620878	Intron	2.09E-06	Brain, thoracicoabdominal ganglion, ovary	x		x	yes	yes
CG31121	FBgn0051121	3R: 20709675	Synonymous coding	2.52E-06	Male accessory glands, crop, hindgut	x	x			
Oatp33Ea	FBgn0032433	2L: 12442228	Synonymous coding	2.62E-06	Midgut, hindgut		x			
Zfp8	FBgn0021875	2R: 20021981	Intron	1.97E-06	Ovary, whole fly, salivary gland	x	x			
Dyb	FBgn0033739	2R: 8331587; 2R: 8331617	Intron	1.89E-06	Virgin and mated spermatheca	x				
CG18659	FBgn0027561	2R: 5019522	Upstream	8.48E-06	Testis, ovary, thoracicoabdominal ganglion, brain	x		x	no	-
CG40006	FBgn0058006	2L: 22594273	Intron	2.82E-06	Head, ovary, eye	x				
alc	FBgn0260972	2R: 5025589; 2R: 5025608	upstream	5.76E-06	Eye, heart, fat body		x			
CLIP-190	FBgn0020503	2L: 17404253	Intron	1.37E-06	Testis, Thoracicoabdominal ganglion, brain	x		x	no	no
CG43658	FBgn0263706	X: 17432030; X: 17432017	Intron	3.92E-06; 8.65E-06	unknown		x	x	no	no
SP1029	FBgn0263236	3R: 25075908	Non-synonymous coding	5.15E-06	Crop, brain, thoracicoabdominal ganglion			x	yes	no
kn	FBgn0001319	2R: 10660559; 2R: 10660661	UTR 3 Prime	2.69E-06	Heart, thoracicoabdominal ganglion, brain			x	yes	no
luna	FBgn0040765	2R: 6932143; 2R: 6932150; 2R: 6932148; 2R: 6937013	Intron	1.01E-06; 1.58E-06; 7.05E-06	Brain, thoracicoabdominal ganglion, mated spermatheca	x		x	yes	yes
CG16947	FBgn0031816	2L: 6462171	Intron	4.65E-06	Thoracicoabdominal ganglion, salivary gland, brain		x	x	yes	no
rhea	FBgn0260442	3L: 8544854	Intron	5.22E-06	Thoracicoabdominal ganglion, heart, salivary gland		x	x	yes	no
px	FBgn0003175	2R: 18432775	Intron	9.83E-06	Hindgut, midgut, crop		x			
CG42255	FBgn0259140	3L: 11798316	Synonymous coding	8.44E-06	Heart					
CG43064	FBgn0262366	3L: 11828422	Non-synonymous coding	6.39E-06	Fat body, tubule, midgut		x			
cv-2	FBgn0000395	2R: 17242683	UTR 3 Prime	1.07E-05	Thoracicoabdominal ganglion, fat body, hindgut		x	x	yes	no
ed	FBgn0000547	2L: 4081644	Intron	1.30E-05	Crop, ovary, male accessory gland	x	x			
Mob2	FBgn0259481	3L: 11521522	Intron	1.00E-05	Eye, thoracicoabdominal ganglion, head, brain			x	yes	yes
CG42540	FBgn0260657	3L: 4564290	Intron	2.44E-05	Midgut, salivary gland, fat body		x			
Sre64B	FBgn0262733	3L: 4613079	Intron	1.52E-05	Ovary, heart, whole fly	x				
Gld	FBgn0001112	3R: 3078190; 3R: 3078191	UTR 3 Prime	1.83E-05	Crop, male accessory gland, hindgut	x	x			
Sfp51E	FBgn0259966	2R: 11080964; 2R: 11081030	Upstream	6.29E-06; 9.80E06	unknown					
pbl	FBgn0003041	3L: 7905142	Upstream	1.01E-05	Salivary gland, ovary, whole fly	x	x			
CR44313	FBgn0265372	2R: 8122817	Upstream	3.15E-05	unknown					
kay	FBgn0001297	3R: 25617567	Synonymous coding	2.04E-05	Crop, hindgut, midgut		x			
Or24a	FBgn0026394	2L: 4164007	Synonymous coding	1.51E-05	Salivary gland		x		yes	
CG8861	FBgn0037676	3R: 511936	Downstream	7.99E-06	Thoracicoabdominal ganglion, brain, eye			x	no	no
Obp47a	FBgn0033573	2R: 6796988	Upstream	2.12E-05	Mated spermatheca, tubule, crop	x	x			
Piezo	FBgn0264953	2L: 8184482	Intron	1.33E-05	unknown				yes	
Rab1	FBgn0031696	2L: 5279698	Synonymous coding	4.37E-06	Ovary, testis, brain	x		x	yes	no
snRNA:U3:2	FBgn0003926	2L: 1433189; 2L: 1433143	Downstream	1.30E-05; 2.37E-05	unknown					
CG42732	FBgn0261698	2R: 6233059	Intron	3.37E-05	Salivary gland, fat body		x			
CG5160	FBgn0031906	2L: 7394198	Intron	1.23E-05	Eye, thoracicoabdominal ganglion, brain		x	x	no	no
CG13170	FBgn0033703	2R: 8126498	Downstream	3.93E-05	Salivary gland, midgut		x			
dysc	FBgn0264066	3L: 13670678	Intron	4.43E-06	unknown					
@ kirre	FBgn0028369	X: 2775990	Intron	4.39E-05	Heart, eye, brain			x	yes	no
l(3)72Ab	FBgn0263599	3L: 15958915; 3L: 15959623	Synonymous coding	1.54E-05	unknown					
T48	FBgn0004359	3R: 22713356	Intron	1.79E-05	Crop, tubule, eye		x			
beat-Ib	FBgn0028645	2L: 1594372	Intron	6.88E-05	Brain, thoracicoabdominal ganglion, head			x	yes	no
cv-c	FBgn0086961	3R: 10260647	Intron	9.22E-06	Hindgut, midgut, fat body		x		yes	

@ Genes also occuring in GWAS for post-mating receptivity (S1 Table 2)

Supplementary Table 2 Genes with SNPs associated to virgin receptivity Table of the variation sites associated to virgin receptivity. The name of the gene, flybase ID, position of the genome of the variants, p-value from simple regression of DGRP2 analyses (*) sorted on most significant, tissue expression as found on flyatlas (') and the characterization in three main themes (") based on tissue expression. In the last two column only the genes from the brain theme are questioned on whether they were found in a previous GWA for olfaction (Arya et al., 2015) and whether they are known to be involved in learning and memory processes.

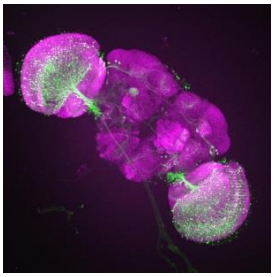
Gene	FlyBase ID	Chromosome position	Gene position	P-value #copulations*	P-value remating latency*	Tissue expression in adult fly'	Sexspecific*	Food related*	Central nervous system*	Hit in olfaction GWA*	Involved in learning and memory?
CG32026	FBgn0052026	3L: 8830967	Synonymous coding	1.32E-02	1.38E-05	Testis	x				
Ir8a	FBgn0052809	X: 2871992; X: 2879465	Intron	7.02E-06	7.55E-04	Heart, eye, brain			x	yes	no
CG17135	FBgn0039135	3R: 7698631	Upstream	7.70E-06	7.55E-04	Heart, whole fly, fat body					
> Dscam	FBgn0261797	3L: 4812885	Intron	1.05E-02	1.17E-03	Crop, brain, hindgut		x		no	yes
CG13144	FBgn0053144	2R: 6662750	Intron	1.29E-02	1.67E-03	Hindgut, brain, salivary gland					
Lerp	FBgn0051072	3R: 22679223	Intron	1.09E-05	1.82E-03	Tubule, midgut, hindgut					
ftz	FBgn0000711	X: 1029649	Intron	8.25E-03	2.89E-03	Salivary gland, eye, thoracicoabdominal ganl					
> CG11170	FBgn0034170	2R: 18605126; 2R: 18065387; 2R: 18065504	Synonymous and nonsynonymous coding	9.64E-03	6.51E-03; 2.28E-02	Ovary, thoracicoabdominal ganglion, fat body	x	x	x	no	-
Ir41c	FBgn0053041	3L: 6645672	Synonymous coding	2.21E-05	6.56E-03	Ovary, whole fly	x				
CG12698	FBgn0052698	X: 3681985	Upstream	-	6.66E-03	Fat body, mated spermatheca, heart		x			
CG18265	FBgn0036723	3L: 17441940	Intron	-	6.73E-03	Brain, thoracicoabdominal ganglion, fat body		x	x	yes	-
CG13905	FBgn0034462	2R: 15533526	Downstream	9.13E-03	7.63E-03	Salivary gland, hindgut, fat body		x			
> Eip78c	FBgn0000568	3L: 17983775	Intron	1.76E-02	7.68E-03	Brain, thoracicoabdominal ganglion, fat body		x	x	yes	no
Ir45	FBgn0052045	X: 16611758	Intron	2.23E-02	8.31E-03	Hindgut, crop, tubule	x	x			
mgl	FBgn0261260	X: 9323447	Intron	-	8.53E-03	Male accessory gland, crop, thoracicoabdominal ganglion	x	x	x	no	no
Plod	FBgn0036147	3L: 11193664	Intron	3.97E-03	1.08E-02	Virgin spermatheca, fat body, mated spermatheca	x				
CG43759	FBgn0264090	X: 18912083; X: 18912330; X: 1891237	Synonymous coding and intron	8.09E-03; 9.21E-03	1.12E-03; 1.20E-03	Ovary, whole fly, salivary gland	x				
Best1	FBgn0040238	3R: 5994385; 3R: 5994851	Intron	2.41E-03; 1.78E-02	1.19E-02	Midgut, hindgut, whole fly	x				
CG43732	FBgn0053732	3R: 14779215	Not known	-	1.21E-02	Hindgut, crop, tubule	x				
gpa	FBgn0034878	2R: 1943970	Intron	-	1.21E-02	unknown					
Evi5	FBgn0262740	X: 11325517	Intron	-	1.37E-02	Testis, brain, whole fly	x		x	no	no
- vn (CG10491)	FBgn0003984	3L: 5811555	UTR 3 Prime	1.30E-02	1.37E-02	Brain, thoracicoabdominal ganglion, salivary gland				no	no
CG43444	FBgn0263392	3L: 2820023	Intron	-	1.47E-02	Eye, heart, tubule				yes	
CG43446	FBgn0053133	3L: 7133997	Upstream	-	1.47E-02	Fat body, thoracicoabdominal ganglion, ovary	x	x	x	yes	no
CR31306	FBgn0051306	3R: 7106997; 3R: 7106711; 3R: 7106712; 3R: 7106920; Intron	Upstream	-	1.47E-02; 1.90E-02;	Testis, whole fly, salivary gland	x				
		3R: 7106984; 3R: 7107157; 3R: 7106822		-	2.08E-02; 2.13E-02	Salivary gland, thoracicoabdominal ganglion, tubule		x	x	no	-
> CG10205	FBgn0033979	2R: 10722008	Downstream	1.43E-02	1.48E-02	Head, whole fly, eye	x		x	yes	-
> Creb3l-17a	FBgn0265784	X: 18263558	Intron	3.30E-02	1.64E-02	Ovary, testis, brain	x		x	no	yes
> shot	FBgn0013733	2R: 9807635; 2R: 9814159; 2R: 9809799; 2R: 9807750 Intron		-	1.77E-02	Crop, mated spermatheca, brain	x		x	yes	yes
> Pdk1	FBgn0026562	2R: 19475752	UTR 5 Prime	-	2.01E-02	Brain, tubule, hindgut	x		x	no	yes
> CG14759	FBgn0037987	3R: 7875160	Upstream	4.01E-02	2.13E-02	Testis, whole fly, salivary gland	x		x		yes
> tau (CG31058)	FBgn0026579	3R: 2347477	Non-synonymous coding	-	2.39E-02	Brain, eye, thoracicoabdominal ganglion				no	yes
CG11200	FBgn0034500	2R: 16191275	Downstream	-	2.47E-02	Crop, fat body, hindgut		x			
- Lilli (CG8817)	FBgn0040111	2L: 2923431	Intron	-	2.82E-02	Testis, heart, eye	x			yes	
CG32085	FBgn0052085	3L: 11660505	Intron	-	3.70E-02	Brain, testis, thoracicoabdominal	x	x	x	no	
Ir47a	FBgn0052047	2L: 2736185	Intron	-	5.22E-02	Crop, hindgut, brain		x	x	yes	
> ATPIB (CG14741)	FBgn0037080	3R: 7893293	Downstream	1.69E-02	-	Thoracicoabdominal ganglion, brain		x	x	no	no

> Genes chosen for their expression in brain tissue

- Genes chosen for their specific known function described in main text

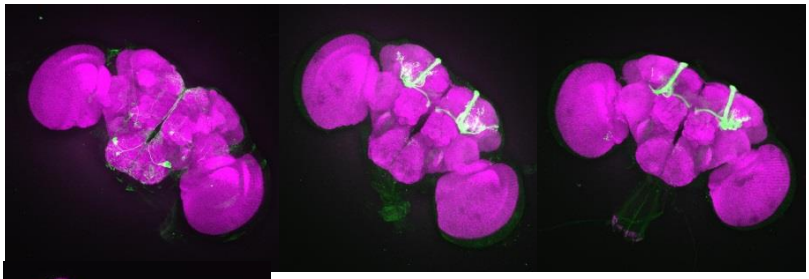
Supplementary Table 3 Genes with SNPs associated to post-mating receptivity Table of the variation sites associated to post-mating receptivity. The name of the gene, flybase ID, position of the genome of the variants, p-value from simple regression of DGRP2 analyses (*) for number of couplations, p-value for latency to first remating (*) sorted on most significant, tissue expression as found on flyatlas (') and the characterization in three main themes (") based on tissue expression. In the last two column only the genes from the brain theme are questioned on whether they were found in a previous GWA for olfaction (Arya et al., 2015)and whether they are known to be involved in learning and memory processes.

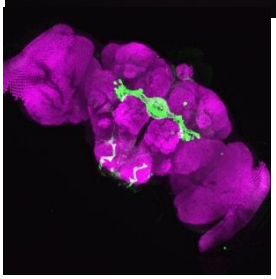
CG10205



Discription of areas: antennal lobe, antennal mechanosensory and motor center, anterior ventrolateral protocerebrum, gall, optic lobe, optic tubercle, saddle.

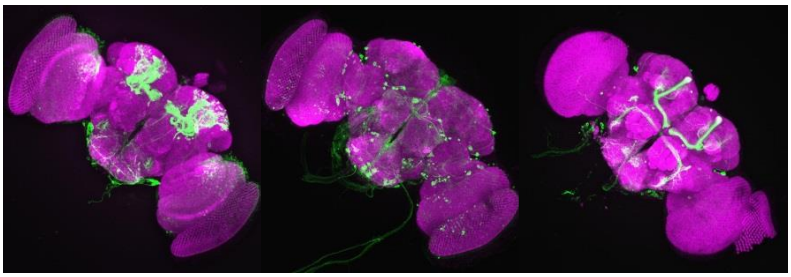
Shot



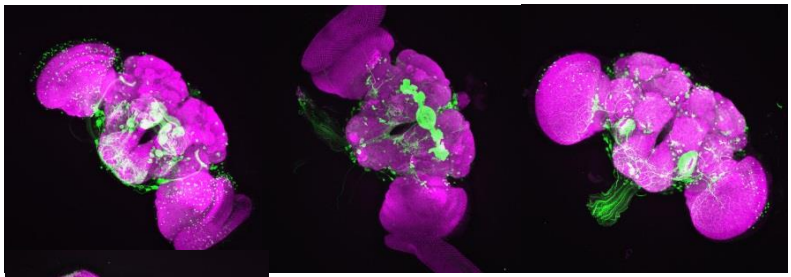


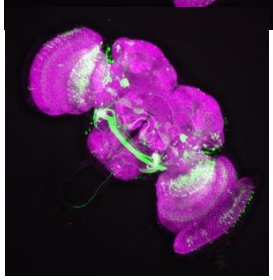
Description of areas: bulb, ellipsoid body, flange, gall, inerior posterior slope, lateral accessory lobe, mushroom body, prow, subesophageal ganglion.

Lilli



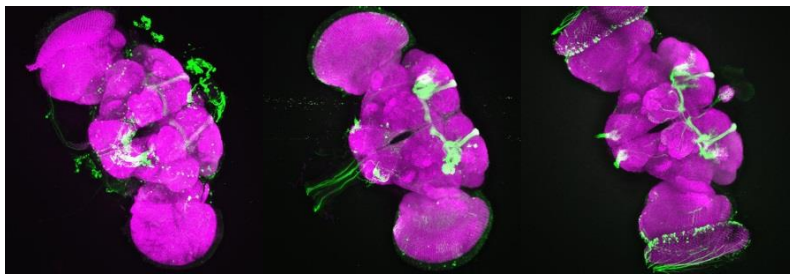
Lilli

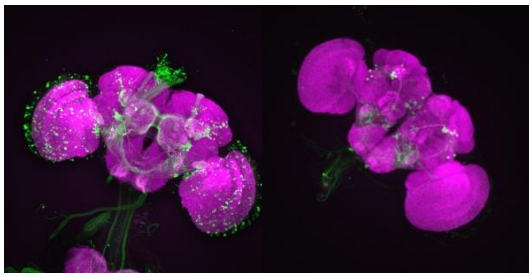




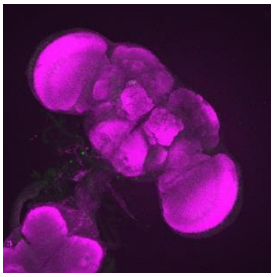
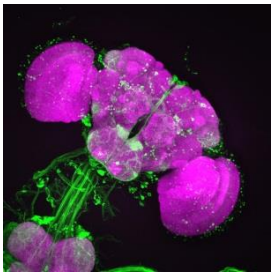
Description of areas: antennal lobe, antennal mechanosensory and motor center, anterior ventrolateral protocerebrum, bulb, ellipsoid body, flange, gall, lateral accessory lobe, mushroom body, optic lobe, posterior lateral photocerebrum, posterior ventrolateral protocerebrum, protocerebral bridge, prow, saddle, subesophageal ganglion, superior intermediate protocerebrum, superior lateral protocerebrum, superior medial protocerebrum.

Eip75b





Description of areas: antennal lobe, antennal mechanosensory and motor sensor, flange, inferior posterior slope, mushroom body, optic lobe, posterior ventrolateral protocerebrum, prow, saddle, subesophageal ganglion, superior clamp, vesta.

Dhc64c  Description of areas: information not given
CrebB-17a  Description of areas: information not given

Supplementary Table 4 FlyLight expression pattern Table with description and visualisation of expression pattern of the candidate genes for post-mating receptivity in *Drosophila melanogaster* brains as found on FlyLight.

Figure 1: Virgin and post-mating receptivity are independently variable phenotypes in the DGRP core set.

Figure panel	Model factors	ChiSquare	p value*	AIC	Explanatory var	Correlation coe	df	t value	p value**
	Model choice								
A	Intercept			2239.70					
	Genotype	**172.520**	**<0.0001**	**2133.20**					
B	Intercept			1544.20					
	Genotype	**165.090**	**<0.0001**	**1445.10**					
C	Intercept			18.97.9					
	Genotype	**151.920**	**<0.0001**	**1812.00**					
D					Virgin latency v	-0.1400	32.0000	-0.771	0.4462
E					Mating frequen	-0.9484	32.0000	-16.917	<0.0001
F					Remating laten	0.2200	32.0000	1.285	0.2080

Figure 4: Knockdown of none of the candidate genes in the central nervous system affects mating frequency.

Figure panel	Model factors	ChiSquare/F	p value*	AIC/RSS	Explanatory var	Estimate	Std. Error (+/-)	t value	p value**
	Model choice				**Post-hoc model values**				
CG102050					UAS vs RNAi	-1.2856	0.3830	-3.357	0.0018
	Intercept			211.31	Gal4 vs RNAi	-0.4675	0.3905	-1.197	0.2385
	Genotype	**10.335**	**0.006**	**204.98**	Gal4 vs UAS	1.0133	0.3522	2.877	0.0064
vn					UAS vs RNAi	-0.3979	0.1061	-3.751	0.0006
	Intercept			70.64	Gal4 vs RNAi	0.0492	0.1168	0.422	0.6753
	Genotype	**17.071**	**0.000**	**57.57**	Gal4 vs UAS	0.4489	0.1157	3.881	0.0004
tau					UAS vs RNAi	-0.3246	0.2771	-1.171	0.2491
	Intercept			191.17	Gal4 vs RNAi	0.5872	0.3318	1.770	0.0845
	Genotype	**7.687**	**0.021**	**187.48**	Gal4 vs UAS	0.8702	0.3573	2.435	0.1945
Dhc64c	**Intercept**			**198.87**					
	Genotype	1.525	0.466	201.34					
CrebB-17a					UAS vs RNAi	-0.8601	0.3295	-2.611	0.1275
	Intercept			208.98	Gal4 vs RNAi	0.2600	0.3332	0.780	0.4398
	Genotype	**10.744**	**0.005**	**202.24**	Gal4 vs UAS	1.1555	0.3803	3.038	0.0042
lilli					UAS vs RNAi	-0.6765	0.3950	-1.713	0.0949
	Intercept			213.93	Gal4 vs RNAi	-0.0256	0.4282	-0.060	0.9525
	Genotype	**4.420**	**0.110**	**213.51**	Gal4 vs UAS	0.6808	0.3790	1.796	0.0797
Pde8					UAS vs RNAi	-1.2818	0.3863	-3.318	0.0020
	Intercept			213.53	Gal4 vs RNAi	-0.1077	0.4312	-0.250	0.8034
	Genotype	**12.934**	**0.002**	**204.59**	Gal4 vs UAS	1.1405	0.3174	3.594	0.0009
CG11170					UAS vs RNAi	-1.5507	0.4060	-3.820	0.0005
	Intercept			230.98	Gal4 vs RNAi	-0.5682	0.4438	-1.2800	0.2079
	Genotype	**13.489**	**0.001**	**221.49**	Gal4 vs UAS	0.9550	0.3560	2.682	0.0105
CG14741					UAS vs RNAi	-0.4413	0.3468	-1.273	0.2108
	Intercept			209.23	Gal4 vs RNAi	0.2097	0.3662	0.573	0.570
	Genotype	**4.255**	**0.119**	**208.97**	Gal4 vs UAS	0.7105	0.3883	1.830	0.0745
Eip75b					UAS vs RNAi	-1.4389	0.3370	-4.270	0.0001
	Intercept			207.12	Gal4 vs RNAi	0.1318	0.3647	0.361	0.7200
	Genotype	**20.328**	**<0.0001**	**190.79**	Gal4 vs UAS	1.5707	0.3592	4.372	<0.0001
shot	**Intercept**			**203.54**					
	Genotype	1.096	0.578	206.45					

Figure 5: Knockdown of none of the candidate genes in the central nervous system affects remating latency.

Figure panel	Model factors	ChiSquare/F	p value*	AIC/RSS	Explanatory var	Estimate	Std. Error (+/-)	t value	p value**
	Model choice				**Post-hoc model values**				
CG102050					UAS vs RNAi	0.1201	2.1216	0.057	0.9549
	Intercept			383.43	Gal4 vs RNAi	-3.2750	1.9450	-1.684	0.1004
	Genotype	**5.225**	**0.073**	**382.20**	Gal4 vs UAS	-3.9490	1.3250	-2.979	0.0050
vn					UAS vs RNAi	7.3280	2.1360	3.431	0.0015
	Intercept			423.45	Gal4 vs RNAi	-1.7250	1.5760	-1.095	0.2802
	Genotype	**20.982**	**<0.0001**	**406.47**	Gal4 vs UAS	-9.2230	2.1780	-4.234	0.0001
tau					UAS vs RNAi	4.7020	2.9890	1.573	0.1242
	Intercept			437.32	Gal4 vs RNAi	-3.8550	1.8510	-2.083	0.0440
	Genotype	**9.333**	**0.009**	**431.99**	Gal4 vs UAS	-8.0840	2.8290	-2.857	0.0068
Dhc64c	**Intercept**			**407.33**					
	Genotype	2.679	0.262	408.65					
CrebB-17a					UAS vs RNAi	0.4610	0.2310	1.995	0.0533
	Intercept			153.55	Gal4 vs RNAi	-0.4113	0.2259	-1.821	0.0761
	Genotype	**13.342**	**0.001**	**144.21**	Gal4 vs UAS	-0.9886	0.2225	-4.444	<0.0001
lilli					UAS vs RNAi	5.5410	2.6030	2.129	0.0398
	Intercept			429.12	Gal4 vs RNAi	-1.1300	1.6470	-0.686	0.4969
	Genotype	**10.180**	**0.006**	**422.94**	Gal4 vs UAS	-6.8450	2.4620	-2.781	0.0081

Figure panel	Model factors	ChiSquare/F	p value*	AIC/RSS	Explanatory vai	Estimate	Std. Error (+/-)	t value	p value**
	Model choice				**Post-hoc model values**				
Pde8					UAS vs RNAi	5.7400	1.2350	4.649	<0.0001
	Intercept			361.29	Gal4 vs RNAi	-1.5380	1.5440	-0.996	0.3256
	Genotype	**24.688**	**<0.0001**	**340.60**	Gal4 vs UAS	-6.9340	1.2700	-5.458	<0.0001
CG11170					UAS vs RNAi	9.3550	2.5850	3.619	0.0008
	Intercept			451.24	Gal4 vs RNAi	-1.2840	1.3890	-0.925	0.3607
	Genotype	**21.782**	**<0.0001**	**433.46**	Gal4 vs UAS	-10.3840	2.6140	-3.973	0.0003
CG14741					UAS vs RNAi	4.9040	2.0260	2.421	0.0205
	Intercept			413.61	Gal4 vs RNAi	-2.0340	1.4800	-1.374	0.1777
	Genotype	**14.205**	**0.001**	**403.41**	Gal4 vs UAS	-6.9740	1.8950	-3.680	0.0007
Eip75b					UAS vs RNAi	0.9328	0.2218	4.206	0.0002
	Intercept			160.59	Gal4 vs RNAi	-0.2506	0.2672	-0.938	0.3540
	Genotype	**22.113**	**<0.0001**	**142.48**	Gal4 vs UAS	-1.2096	0.2160	-5.599	<0.0001
shot	**Intercept**			**398.38**					
	Genotype	4.063	0.131	398.31					

Figure 6: Knockdown of Pde8 in the mushroom bodies decreases mating frequency.

Figure panel	Model factors	ChiSquare/F	p value*	AIC/RSS	Explanatory vai	Estimate	Std. Error (+/-)	t value	p value**
	Model choice				**Post-hoc model values**				
CG102050	**Intercept**			**206.26**					
	Genotype	3.683	0.159	206.58					
vn					UAS vs RNAi	0.7623	0.3846	1.982	0.0546
	Intercept			215.10	Gal4 vs RNAi	-0.0627	0.4199	-0.149	0.8823
	Genotype	**5.703**	**0.058**	**213.40**	Gal4 vs UAS	-0.8071	0.4649	-1.736	0.0905
tau	Intercept			185.79					
	Genotype	1.175	0.556	188.62					
CrebB-17a	**Intercept**			**199.80**					
	Genotype	0.688	0.709	203.11					
lilli	**Intercept**			**197.30**					
	Genotype	1.180	0.554	200.12					
Pde8					UAS vs RNAi	0.3690	0.3084	1.197	0.2391
	Intercept			184.61	Gal4 vs RNAi	0.8026	0.3599	2.230	0.0319
	Genotype	**4.745**	**0.093**	**183.87**	Gal4 vs UAS	0.5429	0.3603	1.507	0.1403
CG11170	**Intercept**			**200.76**					
	Genotype	1.149	0.563	203.61					
CG14741	**Intercept**			**193.73**					
	Genotype	0.507	0.776	197.22					
Eip75b	**Intercept**			**199.52**					
	Genotype	1.820	0.403	201.70					
shot	**Intercept**			**192.57**					
	Genotype	1.836	0.399	194.73					

Figure 7: Knockdown of Pde8 in the mushroom bodies increase remating latency.

Figure panel	Model factors	ChiSquare/F	p value*	AIC/RSS	Explanatory vai	Estimate	Std. Error (+/-)	t value	p value**
	Model choice				**Post-hoc model values**				
CG102050	**Intercept**			**168.72**					
	Genotype	0.699	0.705	172.02					
vn					UAS vs RNAi	-0.6870	0.2504	-2.744	0.0091
	Intercept			166.10	Gal4 vs RNAi	-0.3015	0.3009	-1.002	0.3227
	Genotype	**6.805**	**0.033**	**163.29**	Gal4 vs UAS	0.3984	0.3056	1.304	0.1998
tau	**Intercept**			**170.17**					
	Genotype	1.710	0.425	172.46					
CrebB-17a	**Intercept**			**49.54**					
	Genotype	0.366	0.833	153.18					
lilli	**Intercept**			**165.84**					
	Genotype	3.108	0.211	166.73					
Pde8					UAS vs RNAi	-0.4883	0.2593	-1.883	0.0678
	Intercept			154.38	Gal4 vs RNAi	-0.7372	0.2755	-2.6760	0.0110
	Genotype	**7.438**	**0.024**	**150.94**	Gal4 vs UAS	-0.2297	0.3021	-0.760	0.4521
CG11170	**Intercept**			**173.09**					
	Genotype	0.793	0.673	176.30					
CG14741	**Intercept**			**169.13**					
	Genotype	3.316	0.191	169.81					
Eip75b	**Intercept**			**168.62**					
	Genotype	2.419	0.298	170.20					
shot	**Intercept**			**456.62**					
	Genotype	3.373	0.185	466.24					

*Log likelihood ratio test **Test statistics (contingency table convert)

Supplementary table 5 Detailed statistics All models in this table were performed using R version 3.2.2. All data that complied with the rules of normality and homogeneity were tested with mixed effects models and the output is shown here with t- and p-values. When either assumption could not be satisfied, the data were log transformed. When applicable a random effect for date was added to the models.